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Short communication

Synthesis of an immobilized *Bombyx mori* pheromone-binding protein liquid chromatography stationary phase☆

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Abstract

The pheromone-binding protein from the silkworm moth, *Bombyx mori* (BmorPBP) has been covalently bonded to a liquid chromatographic stationary phase. The resulting column was evaluated using radiolabeled bombykol and the immobilized protein retained its ability to bind this ligand. The data also demonstrate that the BmorPBP column was able to distinguish between four compounds, and rank them in their relative order of affinity for the protein from highest to lowest: bombykol > bombykal > 1-hexadecanol > (*Z,E*)-5,7-dodecadien-1-ol, and that the immobilized BmorPBP retained its pH-dependent conformational mobility.

The results of this study demonstrate that pheromone-binding protein from the silkworm moth, *Bombyx mori* and an odorant binding protein (OBP) obtained from the female mosquito *Culex quinquefasciatoes* have been immobilized on a silica support with retention of ligand-binding activity. The data indicate that proteins from non-mammalian organisms can be used to create liquid chromatography affinity columns.

Keywords: Affinity chromatography; Screening; Conformational mobility; Odorant binding protein

1. Introduction

Life on the insect scale is a chemical dominion; therefore, insects rely on pheromones to communicate, and their senses of smell and taste dominate the search for both plant and animal hosts. Pheromone and odorant binding proteins (PBPs and OBPs) are a family of small soluble proteins (15 kDa) containing six α-helices enclosing a hydrophobic ligand-binding pocket [1]. These proteins appear to be the major link between the external universe of chemical signals to which insects are sensitive, and the odorant receptors (ORs) embedded in antennal neurons [2,3].

The identification and characterization of OBPs has led to speculation that these proteins may be fruitful research targets in the design of novel insect control tactics, particularly against disease-transmitting mosquitoes [1]. One approach to the development of a screen for potential attractants, pheromones, and repellents is the development of liquid chromatographic stationary phases containing the immobilized OBP or PBP target. The resulting chromatography columns can then be used in online screens. This approach has been suggested by the use of immobilized protein-based liquid chromatography columns in protein-binding studies, which have been prepared from mammalian serum albumins and α_1-acid glycoprotein (for a recent review see Ref. [4]). Drug receptors and transporters have also been immobilized and used in affinity screens [5]. However, to our knowledge, insect-derived binding proteins have not been immobilized and used in affinity chromatography studies.

This manuscript reports the initial synthesis and characterization of a liquid chromatography stationary phase containing an immobilized PBP obtained from the silkworm moth, *Bombyx mori*, the BmorPBP. A second insect-derived prortein, an odorant binding protein (OBP) obtained from the female mosquito *Culex quinquefasciatoes*, has also been immobilized with retention of binding activity.

☆ Mention of commercial products does not constitute an endorsement by USDA.

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2. Experimental

2.1. Reagents

2,4,6-Trichloro-1,3,5-triazine (cyanuric chloride), 3-aminopropyltriethoxysilane (APTS), sodium phosphate monohydrate, potassium chloride and glycine ethyl ester were obtained from Aldrich Chemical Company (Milwaukee, WI). Dibasic sodium phosphate (Anhydrous Sigma Ultra) was purchased from Sigma Chemical Company (St. Louis, MO).

2.2. Expression of pheromone and odorant binding proteins

The BmorPBP was produced by recombinant techniques following a previously described method [6] and the odorant binding protein (OBP) obtained from the female mosquito *Culex quinquefasciatoes* was also obtained following previously described procedures [7].

2.3. Immobilization of BmPBP

The immobilization was accomplished following a previously described procedure used with bovine serum albumin [8] in which 50 mg of silica (5 μm, 300 Å) from Chrom Expert (Sacramento, CA, USA) was activated with cyanuric chloride, packed in a HR-5/2 glass column to yield a 3 mm × 5 mm (i.d.) chromatographic bed and the resulting column was linked to a peristaltic pump (Minipulse 2, Rainin, Woburn, MA, USA). A solution of phosphate buffer (50 mM, pH 7.0) was delivered through the column at 50 μl/min for 1 h, followed by 1.6 mg of BmorPBP in 1 ml phosphate buffer (50 mM, pH 7.0), which was recycled through the column at 25 °C for 8 h. The column was then washed with an aqueous glycine ethyl ester solution (1%, w/w, pH 6.7) at 25 °C for 2 h, in order to cap any unreacted groups. A second batch of activated silica was only capped with glycine ethyl ester and used to create a control column.

2.3.1. Immobilization of OBP

The immobilization was carried out following the protocol for the BmPBP. The cyanuric acid activated silica was packed in a HR-5/2 glass column to yield a 3 mm × 5 mm (i.d.) chromatographic bed and the resulting column was linked to a peristaltic pump (Minipulse 2, Rainin, Woburn, MA). A solution of phosphate buffer (50 mM, pH 7.0) was delivered through the column at 50 μl/min for 1 h, followed by 1.5 mg of OBP in 1 ml phosphate buffer (50 mM, pH 7.0), which was recycled through the column at 25 °C for 4 h. The column was then washed with an aqueous glycine ethyl ester solution (1%, w/w, pH 6.7) at 25 °C for 2 h, to cap any unreacted groups and sequentially washed with 5 ml of each of the following solutions: phosphate buffer (50 mM, pH 7.0); phosphate buffer (25 mM, pH 7.0) containing 25 mM NaCl; deionized water; phosphate buffer (50 mM, pH 7.0). The column was then stored at 4 °C until further use.

2.4. Protein assay

A Micro BCA Protein Assay Kit® from Pierce (Rockford, IL, USA) was used to determine that 1.63 nmol of BmPBP protein had been immobilized per mg of silica and 1.21 nmol of OBP protein had been immobilized per mg of silica.

2.5. Frontal chromatographic studies

The BmorPBP column (or the control column) was placed in a chromatographic system consisting of a LC-10AD isocratic HPLC pump purchased from Shimadzu (Columbia, MD, USA) and an IN/US system β-ram Model 3 on-line scintillation detector from IN/US (Tampa, FL, USA) with a dwell time of 2 s and running Laura Lite 3 software. The mobile phase consisted of phosphate buffer (50 mM, pH 7.0) delivered at 0.2 ml/min at room temperature. Between every injection the column was washed for 18 h with phosphate buffer (50 mM, pH 4.5) containing 50 mM potassium chloride and 7% methanol, followed by an 18 h wash with phosphate buffer (50 mM, pH 7.0).

The compounds used in this study were (*Z,E*)-10,12-hexadecadien-1-ol (bombykol), 1-hexadecanol and (*Z,E*)-5,7-dodecadien-1-ol which were provided by Dr. Ashot Khrimian (USDA-ARS CAIBL, Beltsville, MD, USA), (*Z,E*)-10,12-hexadecadien-1-al (bombykal) which was synthesized from bombykol as previously described [9] and [^{3}H]-bombykol which was obtained from the reduction of bombykal by tritium containing reagents at Amersham Life Sciences (Piscataway, NJ, USA). The binding affinity of [^{3}H]-bombykol was determined with a series of concentrations: 80, 100, 120, 150, 200, 250, and 300 pM.

2.6. Zonal chromatographic studies

The studies were carried out with CquiOBP column using the chromatographic system Series 1100 liquid chromatography/mass selective detector, LC/MSD (Agilent Technologies, Palo Alto, CA, USA) equipped with a vacuum de-gasser (G 1322 A), a binary pump (1312 A), an autosampler (G1313 A) with a 20 μl injection loop, a mass selective detector, MSD (G1946 B) supplied with atmospheric pressure ionization electrospray (API-ES) and an on-line nitrogen generation system (Whatman, Haverhill, MA, USA). The chromatographic system was interfaced to a 250 Mhz Kayak XA computer (Hewlett–Packard, Palo Alto, CA, USA) running ChemStation software (Rev B.10.00, Hewlett–Packard). The mobile phase was deionized water for chiral separation of D,L-lactic acid and ammonium acetate buffer (50 mM, pH 5.0–8.0) for pH dependence and ammonium bicarbonate 10 mM for displacement studies, respectively. The flow rate was 0.2 ml/min, and the experiments were carried out at 37 °C.

3. Results and discussion

In the current study, BmorPBP was initially recycled through the activated column for 4 h and 1.07 nmol of BmorPBP was immobilized per mg of silica support (53% of starting material) which was similar to what was obtained with BSA in which 1.4 nmol of BSA were immobilized in 2 h [8]. In this study, when the recycling time was increased to 8 h the amount of protein immobilized increased to 1.63 nmol per mg of silica

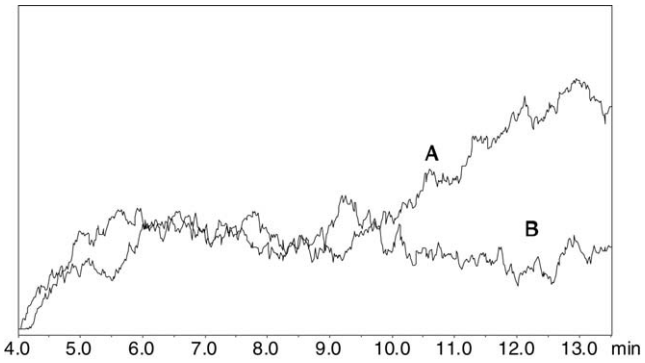

Fig. 1. Frontal chromatogram of a 10 ml sample of 80 pM [^{3}H]-bombykol in phosphate buffer (50 mM, pH 7.0) at 0.2 ml/min at room temperature on the β-RAM radioflow on the BmorPBP column (A) and control column (B).

support (81% of starting material); additional reaction time did not improve the results. The results indicate that the kinetics of the immobilization reaction was slower with BmorPBP relative to BSA and that protein immobilizations must be individually optimized.

Frontal affinity chromatography techniques, which have been recently reviewed [5], were used to study the binding of the marker ligand, [^{3}H]-bombykol, and test ligands to the immobilized protein.

The chromatographic trace produced by the application of 80 pM [^{3}H]-bombykol on the BmorPBP column contained both front and plateau regions (Fig. 1A). The midpoint of the breakthrough curve occurred at an 11.5 min. When the same sample was chromatographed on the control column, the resulting trace did not contain front and plateau regions, and the retention time, calculated at half-height to detector saturation, was 2.8 min (Fig. 1B). The results indicate that non-specific retention due to the chromatographic backbone is not a significant factor in the retention of [^{3}H] bombykol on the BmorPBP column and that the data obtained with this column reflects binding interactions between bombykol and the immobilized BmorPBP.

The sequential application of increasing concentrations of [^{3}H]-bombykol produced a series of frontal curves that were used to calculate the binding affinity of [^{3}H]-bombykol for the immobilized BmorPBP, expressed as the dissociation constant (K_d), as well as the number of active binding sites [P] on the immobilized protein. The data was processed using a one-site non-linear regression model [5] and the calculated K_d value was 80 ± 22 pM, $r^2 = 0.925$ with $[P] = 271 \pm 29$ fmol. No displacement of [^{3}H]-bombykol was observed on the control column.

Competitive displacement studies with 200 pM [^{3}H]-bombykol as marker ligand were carried out to determine the selectivity of the immobilized BmorPBP. The retention time of the marker alone was 9.2 min. The addition of 1 μM bombykol to the mobile phase displaced the retention of the marker by 5.9 to 3.3 min, the dead volume of the column. Using the effect of bombykol as the relative standard, 1 μM concentrations of three test compounds were added to the mobile phase, bombykal, 1-hexadecanol, and (*Z,E*)-5,7-dodecadien-1-ol. The greatest effect was produced by bombykal, which reduced the retention of [^{3}H]-bombykol by 3.2 to 6.0 min, about 50% of the effect produced by 1 μM bombykol. This is consistent with the observations by Pophof, where bombykal was shown to elicit some binding to the BmorPBP [10]. The addition of 1-hexadecanol, a saturated bombykol, produced a displacement of [^{3}H]-bombykol, albeit by only 1 to 8.2 min, indicating that 1-hexadecanol had a lower affinity for BmorPBP relative to bombykal. The addition of (*Z,E*)-5,7-dodecadien-1-ol to the mobile phase resulted in no change in the retention of [^{3}H]-bombykol indicating that this compound had no affinity for the BmorPBP. This is consistent with the fact that (*Z,E*)-5,7-dodecadien-1-ol is the main component of the Siberian moth pheromone [11]. These results demonstrate that the BmorPBP column was able to distinguish between four compounds, and rank them in their relative order of affinity for the protein from highest to lowest: bombykol > bombykal > 1-hexadecanol > (*Z,E*)-5,7-dodecadien-1-ol, which was the expected order based upon structure and previous binding studies (unpublished data).

The data from this study demonstrate that the BmorPBP has been successfully immobilized onto a silica-based stationary phase through a covalent linkage. The immobilized protein retained its ability to bind a known ligand, bombykol, and to differentiate between compounds with some or no affinity for the BmorPBP. To our knowledge this is the first report of the development of a liquid chromatography affinity support containing an immobilized protein obtained from an insect.

However, the results also demonstrate that it takes about 36 h to regenerate the bombykol binding affinity of the immobilized BmorPBP and that the initial wash required lowering the pH of the mobile phase to 4.5 followed by a second wash at pH 7.0. These steps are consistent with the observation that a pH 4.5 the BmorPBP exists in a conformational state that does not bind ligands and at pH ≥ 6.5 the protein exists in a second conformation to which ligands can bind [1,6,12]. The chromatographic data suggests that the immobilized BmorPBP is capable of undergoing the same conformational changes, albeit at a much slower rate.

The regeneration of the binding affinity of the immobilized BmorPBP is the rate-limiting step in the use of this column for online screening of chemical and biological libraries. A key step in the optimization of this column is to reduce the time required to regenerate the binding affinity. Initial studies of the effect of the ionic strength of the mobile phase on the binding of bombykol to BmorPBP have demonstrated that an increase in ionic strength from 50 to 200 mM decreased the retention of bombykol (data not shown). This approach as well as the addition of organic modifiers to the mobile phase is currently under investigation and the results will be reported elsewhere.

The general applicability of this approach has been tested using an odorant binding protein (OBP) obtained from the female mosquito *Culex quinquefasciatoes* [12]. The OBP column contained 1.21 nmol protein per mg silica (60% of the applied OBP) and the binding activity of the immobilized OBP was assessed using L-lactic acid, which has been established as a mosquito attractant [13]. Zonal chromatographic studies using D,L-, D- and L-lactic acid established that the OBP column enantioselectively retained L-lactic acid (Fig. 2), indicating that

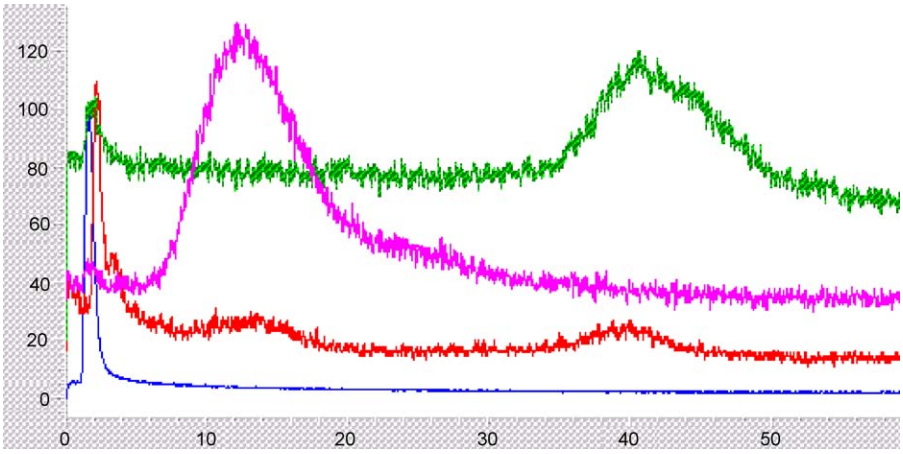

Fig. 2. Zonal chromatographic studies carried out on a column containing an immobilized odorant binding protein obtained from the female mosquito *Culex quinquefasciatoes* where the red chromatographic trace was produced by 500 nM D,L-lactic acid; green trace by 250 nM L-lactic acid; purple trace by 250 nM D-lactic acid and blue trace by the injection of 500 nM D,L-lactic acid on a control column not containing the immobilized odorant binding protein. Studies were carried out using a Series 1100 liquid chromatography/mass selective detector, LC/MSD (Agilent Technologies, Palo Alto, CA, USA) equipped with a 20 μl injection loop, the mobile phase was deionized water, the flow rate was 0.2 ml/min, and the experiments were carried out at 37 °C.

the immobilized protein retained the ability to selectively bind ligands.

The data from these studies demonstrate that insect-derived odorant and pheromone-binding proteins can be immobilized on silica-based liquid chromatographic stationary phases with retention and used as affinity screens. The optimization of these columns and the expansion of their applications will be reported elsewhere.

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